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# CHARACTERIZATION OF SYNTHETIC CARRIER AMPHOLYTES FOR ISO-ELECTRIC FOCUSING

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## SUMMARY

The synthesis of carrier ampholytes suitable for isoelectric focusing is described. The mixture of hexamethylenetetramine (HMTA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA) ampholytes closely resembles commercial Ampholine, and covers the pH range 3–9.5. We have been able to detect focused ampholytes in a gel slab, taking advantage of their different refractive indices, and to assess their relative amounts along the pH gradient.

PEHA ampholytes contain up to 20% of chromophoric structures, with two UV peaks at 368 and 315 nm, in a pH-dependent equilibrium, associated with a very weak nitrogen function having a p$K$ of 1.1. This could be the p$K_6$ of the last amino group in PEHA. However, NMR spectra failed to reveal any nitrogen heterocyclic structure formed during the synthesis.

This mixture of ampholytes exhibits good conductivity, produces smooth pH gradients and allows sharp protein separations in the pH range 3–9.5. Their synthesis is very easy and their cost is extremely low. Their availability should make feasible large-scale preparative isoelectric focusing, and attract more interest to continuous-flow techniques, where large amounts of ampholytes are required.

## INTRODUCTION

The development of the technique of isoelectric focusing (IEF) represents a major breakthrough in the field of high-resolution separations of amphoteric molecules. IEF is an equilibrium method in which amphoteric substances are segregated according to their isoelectric points (p$I$) in a pH gradient. In a series of theoretical papers, Svensson[1-3] laid the foundations of IEF in its present form: he introduced the idea of developing a "natural" pH gradient from amphoteric molecules having closely spaced p$I$s and high conductivities. Under an electric field, these ampholytes would be distributed according to their p$I$ values to form a pH gradient increasing monotonically from the anode to the cathode.

In the early work on IEF, an extensive search for commercially available ampholytes that might be useful as carrier ampholytes was not very successful.

Svensson[2] published a list of these substances, but was unable to find suitable am-
pholytes with good conductivity and buffering capacity in the pH range 3.9–7.3. In
subsequent experiments, Vesterberg and Svensson[4] were able to obtain, by hydrolysis
of proteins, oligopeptides with different p$I$ values and rather good electrochemical
properties. However, even these preparations lacked satisfactory carrier ampholytes
in the pH range 4.5–6, and they also had the drawback of exhibiting properties that
resembled those of proteins too closely.

Efforts were then directed toward synthetic processes. Vesterberg[5] synthesized
a mixture of a large number of homologues and isomers of aliphatic polyamino poly-
carboxylic acids with different p$K$s and p$I$s closely spaced in the pH range 3–10. Their
synthesis involves the coupling of propionic acid residues to polyethylene polyamines.
Carrier ampholytes with suitable properties are obtained when appropriate amounts
of acrylic acid are allowed to react with different polyethylene polyamines in water at
70° until all of the acrylic acid has been consumed. The synthesis proceeds via an
anti-Markovnikov addition after the Michael reaction and therefore $\beta$-amino acids are
obtained. These carrier ampholytes, encompassing the pH range 3–10, are commer-
cially available from LKB (Stockholm, Sweden) under the trade-name Ampholine.

By utilizing polyamines with the amino groups more than three methylene
groups apart, Lundblad et al.[6] were able to synthesize more alkaline ampholytes,
with p$I$ values up to 11.1, thus extending the range for protein separations. Some
more acidic ampholytes have also been synthesized, and have proved useful[7] for pro-
tein separations down to pH 2.5. Recently, Vinogradov et al.[8] described a modification
of the Vesterberg synthetic procedure, and obtained suitable carrier ampholytes in
the pH range 4–8 by coupling pentaethylenehexamine with acrylic acid.

At present, most laboratories use isoelectric focusing routinely on an analytical
scale, especially in gel media, because of the simplicity, reproducibility and speed of
the method. We feel that progress in preparative isoelectric focusing, especially when
using continuous-flow techniques[9], has been severely hampered by the high cost of
Ampholine and by difficulties in recovering them after a run, especially when using
sucrose density gradient stabilized columns. From this point of view, it is surprising
that it took so long to "rediscover" Vesterberg's synthetic procedure, and that so far
only few laboratories have adopted it.

In an attempt to attract more interest to "home-made" carrier ampholytes and
to encourage further the preparative aspects of IEF, we describe here the synthesis
of carrier ampholytes suitable for isoelectric focusing and report some of their
physico-chemical properties. In the following paper, we describe a simple and
reproducible method for fractionating the synthetic mixture in narrow pH ranges
covering one or two pH units.

## MATERIALS AND METHODS

Hexamethylenetetramine (HMTA), triethylenetetramine (TETA), tetraethyl-
enepentamine (TEPA) and pentaethylenehexamine (PEHA) were obtained from
Hoechst Italia (Milan, Italy) and acrylic acid, acrylamide and N,N'-methylenebis-
acrylamide (Bis) were obtained from Merck-Schuchardt (Munich, G.F.R.). Bis was
recrystallized from acetone and the acrylamide from chloroform, as described
by Loening[10].

UV–visible spectra were measured with a Cary 118 spectrophotometer (Varian, Palo Alto, Calif., U.S.A.). Fluorescence spectra were obtained with a Perkin-Elmer Model MPF-2A fluorescence spectrometer, fitted with a Hitachi recorder. Nuclear magnetic resonance (NMR) spectra were recorded with a Perkin-Elmer Model R 12 NMR spectrometer at 60 MHz. Optical rotatory dispersion (ORD) spectra were obtained with a Jasco ORD/UV 5 spectropolarimeter.

Isoelectric focusing was performed in a gel slab, using the LKB 2117 Multiphor and a pulsed power supply, as described by Righetti and Righetti[11]. Scans of pictures of focused ampholytes in a gel slab were obtained with a chromoscan from Joyce, Loebl & Co. (Burlington, Mass., U.S.A.) equipped with an automatic integrator. pH gradients were measured with a Radiometer pH meter fitted with a combination microelectrode, in $1 \times 0.5$ cm gel segments, eluted with 0.3 ml of 10 m$M$ sodium chloride solution, at room temperature.

Conductimetric studies were carried out with a conductimeter from Instrumentation Laboratory, in a 2-ml cell thermostated at 25°.

## RESULTS

### Reagent distillation

Acrylic acid has to be distilled in order to remove the polymerization inhibitor (200 ppm of $p$-methoxyphenol). This is carried out under a stream of nitrogen under reduced pressure. Usually only the amount of acrylic acid that is needed for the coupling reaction with the polyamine is distilled. However, we have also tried batchwise distillations, immediately using the amount of acrylic acid needed and storing the remainder in a nitrogen atmosphere in a freezer. Under these conditions, we have not observed the formation of self-polymers (polymethacrylate).

Perhaps the most cumbersome operation during the synthesis of carrier ampholytes is the distillation of PEHA. This is a necessary step, in order to remove yellow compounds present in the commercial product. One could avoid distilling the various polyamines and then remove the chromophoric compounds present by repeated charcoal treatment, after the synthesis, as described by Vinogradov et al.[8]. However, we have observed that, in this case, the amount of "coloured" carrier ampholytes obtained is greatly enhanced, and that they tend to undergo a browning process upon storage. Fig. 1 depicts the distillation set up for purification of PEHA. This material has a very high boiling point and under a vacuum of 500 $\mu$mHg it distills in the temperature range 200–290°. The only efficient process was to use a sand-bath, with a multiple bunsen burner, and an extra bunsen burner for the Vigreux column. The vacuum pump is protected by a Fresenius tower containing concentrated sulphuric acid, followed by a calcium chloride trap. During the distillation process, the nitrogen used should be oxygen-free in order to prevent further oxidation of PEHA, which is not cooled in the condenser so as to avoid an increase in viscosity. To achieve this, the gas from the nitrogen tank flows through a catalytic burner (copper wire at 450°) and is subsequently cooled in a "serpentine" (see Fig. 1). This process effectively reduces the absorption spectrum of PEHA, although it does not completely eliminate the peak at 290 nm and a shoulder at 330 nm. As a comparison, free acrylic acid does not show any appreciable chromophore above 300 nm (see Fig. 2). The distillation of TETA, TEPA and acrylic acid is a much easier process, because of their lower boiling
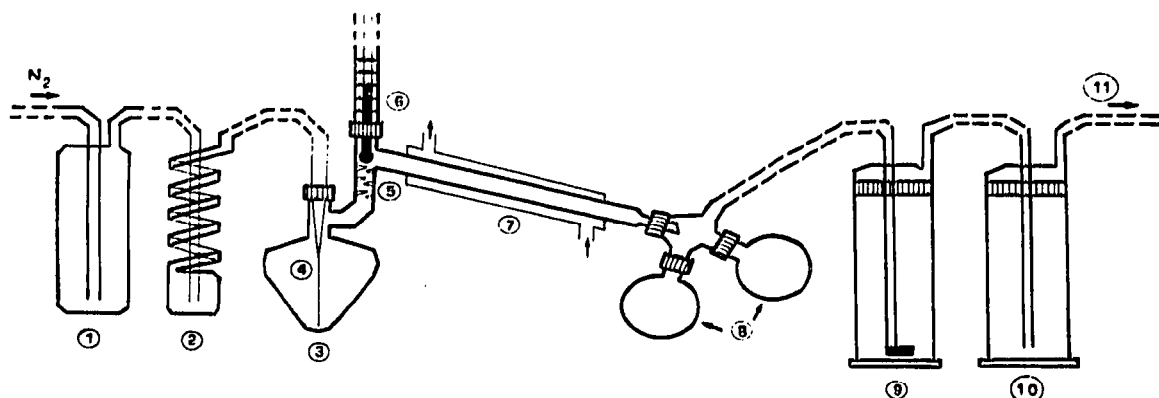
Fig. 1. Scheme of the distillation procedure. 1, Copper wire catalyst, kept in an oven at 450°; 2, cooling "serpentine"; 3, distillation flask; 4, capillary for nitrogen flushing; 5, Vigreux column; 6, thermometer; 7, condenser; 8, collection flasks; 9, Fresenius tower with conc. $H_2SO_4$; 10, $CaCl_2$ trap; 11, connection to the vacuum pump. The shaded regions represent ground-glass joints.
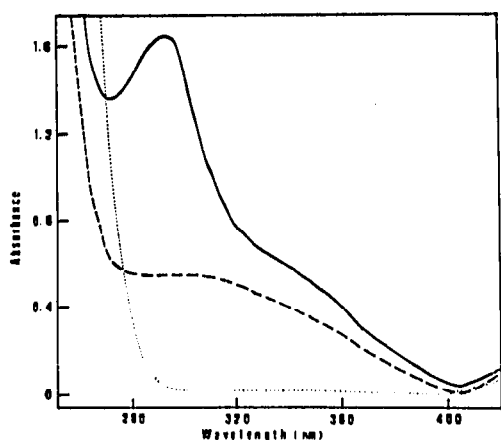


Fig. 2. UV spectra obtained with a Cary 118 spectrophotometer. Solid line: spectrum of commercial PEHA. Broken line: PEHA spectrum after distillation. Dotted line: acrylic acid spectrum. All samples were 1 % (v/v) solutions.

points. HMTA was used as such, and not recrystallized, because its solutions did not show any appreciable chromophore in the 250–500 nm range.

*Synthesis of carrier ampholytes*

We have followed, as a general procedure, that outlined by Vesterberg[5]. The reaction was carried out in a two-necked flask, equipped with a capillary for nitrogen flushing and with a burette for the addition of acrylic acid, fitted with a side-arm as a gas outlet. The polyamine is diluted with water to 0.15 $M$ in the flask, cooled and de-gassed. Then acrylic acid is added dropwise from a burette, with continuous stirring, over a period of 60 min so as to provide the desired nitrogen:carboxyl ratio. The reaction is exothermic, and during the addition it is critical to flush the system with

oxygen-free nitrogen in order to prevent yellowing and browning of the products. For this purpose, we use nitrogen purified through a catalytic burner, as shown in Fig. 1. After the addition of acrylic acid, the flask is stoppered, sealed with Parafilm and transferred to a Dubnoff shaker, thermostated at 70°, for 16–20 h. The reaction mixture is then cooled at room temperature and enough distilled water added to make a 40% (w/v) solution of the carrier ampholytes.

In agreement with the reports of Vesterberg[5] and Vinogradov et al.[8], we also found that the best carrier ampholytes in the pH range 3–10 were obtained with a nitrogen:carboxyl ratio of 2:1. Therefore, in coupling the various polyamines with acrylic acid, we always used this ratio. Under these conditions, when samples of the reaction mixture were tested for unreacted acrylic acid with potassium permanganate, as described by Vesterberg[5], virtually no free acrylic acid could be detected. In a few experiments with PEHA, we increased the amount of acrylic acid added, decreasing the nitrogen:carboxyl ratio to 1.5:1. Under these conditions, approximately 70% of the ampholyte population is shifted in the p/ range 3–5.5, and more than 10% of the acrylic acid remains unreacted. This type of ampholyte can be useful to reinforce the acid side of the pH range, provided that the free acrylic acid is removed, for instance, by electrolysis (see the following paper). A further increase in the amount of acrylic acid added, to a nitrogen:carboxyl ratio of 1:1, leads to ampholytes of no practical value, and leaves more than 25% of unreacted acrylic acid (see Fig. 3).
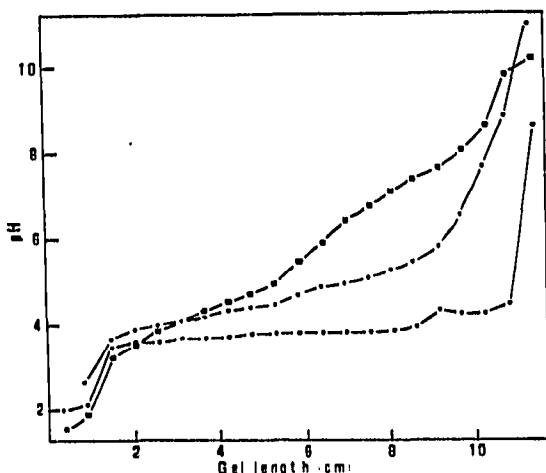


Fig. 3. pH gradients obtained with PEHA ampholytes in a polyacrylamide gel slab. PEHA ampholytes synthesized with a nitrogen:carboxyl ratio of: ■, 2:1; ★, 1.5:1, ●, 1:1. In the last case, no useful ampholytes are obtained.

The ampholytes thus synthesized are yellow-orange in colour. These coloured compounds are, in fact, amphoteric and they focus in the pH range 3–10. With PEHA ampholytes focused in a gel slab, we were able to detect 8–10 yellow focused bands, and we could collect and characterize six of them, having p/s of 2.8, 3.4, 4.5, 5.5, 6.2 and 8.5. They represent up to 20% of the entire ampholyte population. While they do not appear to interfere with protein separations and staining in polyacryl-

amide gels, they can in any event be substantially reduced by repeated (3–4 times) treatment with activated charcoal (2 g per 100 ml of 20 % ampholyte solution). The mixture is de-gassed, kept under vacuum and heated at 80–90° for 10 min. After cooling and filtering, the ampholytes are stored frozen in brown bottles.

## TETA ampholytes

TETA ampholytes, synthesized as described above, with a nitrogen:carboxyl ratio of 2:1, were used to polymerize a polyacrylamide gel slab to a final concentration of 5 % of acrylamide and 2 % of ampholyte. The gel was photopolymerized with riboflavin, as described by Vesterberg[12]. At equilibrium, taking advantage of the different refractive indices of ampholytes along the gel length, we were able to reveal them by photography, as shown in Fig. 4. The rope-like structures are clusters of focused ampholytes, and the valleys in between are regions with low concentrations of or
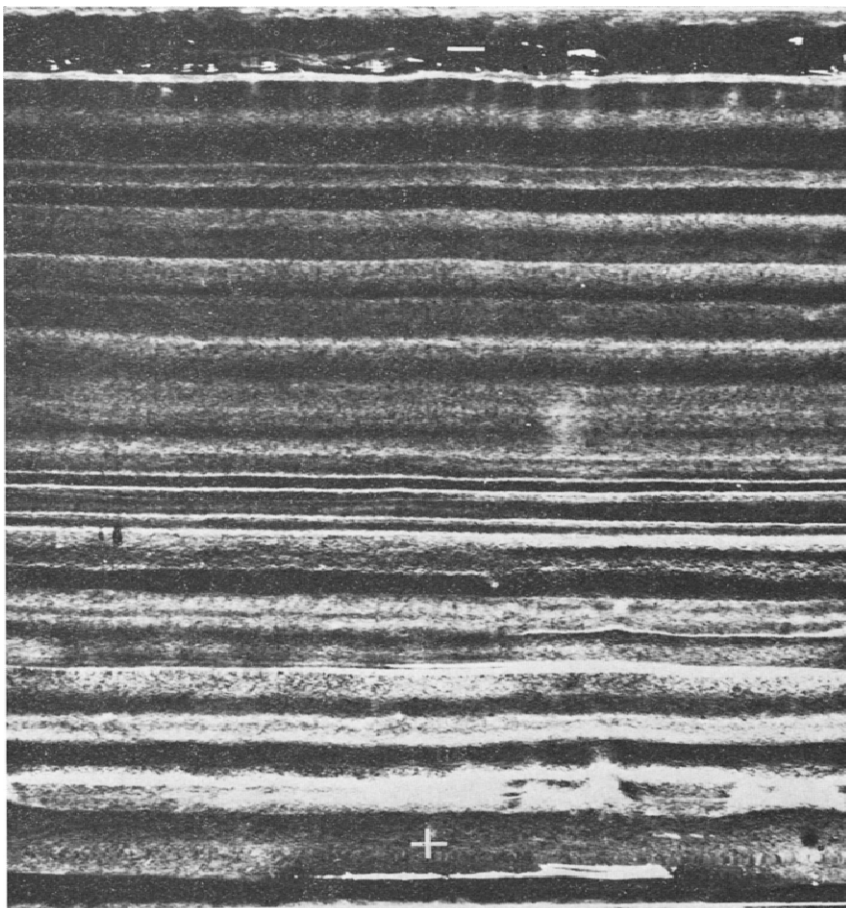


Fig. 4. TETA ampholytes focused in a gel slab. At equilibrium, the gel was photographed against a black background with side illumination. The rope-like structures are clusters of focused ampholytes. This is a picture of a transparent, unstained gel and the ampholytes are detected on the basis of different refractive indexes. The anode (+) and the cathode (−) are marked.

almost no ampholytes. The scan of it in Fig. 5 reveals the ampholyte clusters and the large gaps in between (indicated by arrows). From both figures it is evident that TETA ampholytes are rather poor along the whole of the pH gradient, as there are too few species and too many gaps between them.
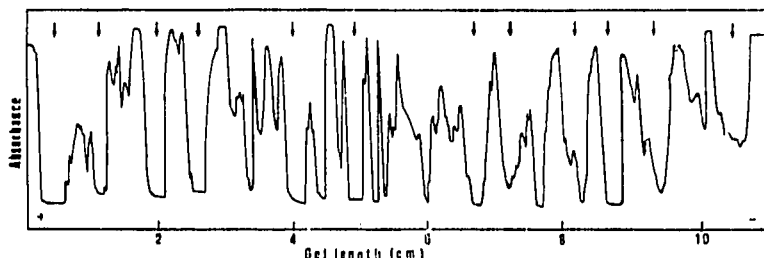


Fig. 5. Scan of focused TETA ampholytes. This scan was made on the print of Fig. 4, with a Joyce, Loebl & Co. chromoscan. The gel polarity is indicated by a + sign (anode, left-hand side) and a − sign (cathode, right-hand side). The arrows indicate gel regions of ampholyte gaps.

Similar conclusions can be drawn from Fig. 6, which gives the pH gradient, the conductance and the UV profiles at 280 and 360 nm obtained by focusing TETA ampholytes in a gel slab. It can be seen that the pH gradient is very uneven and that there are conductivity gaps in several regions of the gel. Therefore, TETA ampholytes do not appear to be suitable for isoelectric focusing, as reported by Vesterberg[5].
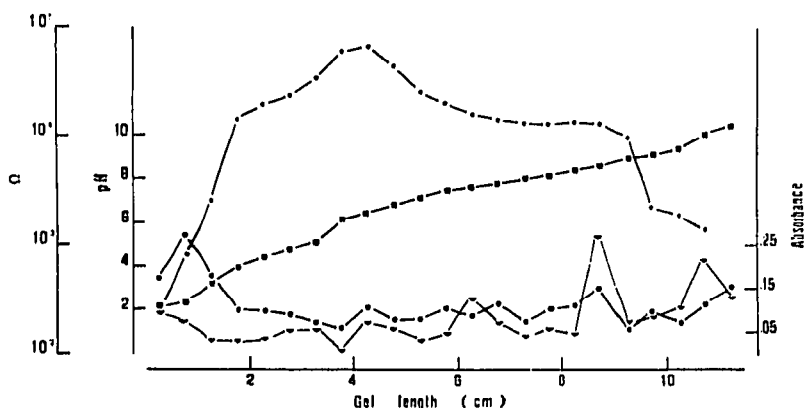


Fig. 6. Conductance (★—★) and pH (■—■) courses and UV profiles at 360 nm (▼—▼) and 280 nm (●—●) of focused TETA ampholytes in a gel slab. For pH determinations, segments of 1 × 0.5 cm were cut. For conductimetry and UV absorbances, gel segments of 4 × 0.5 cm were cut and eluted in 3 ml of distilled water.

*TEPA ampholytes*

A gel slab containing 2% of TEPA ampholytes and 5% of acrylamide was polymerized and subjected to electrofocusing. A picture of the focused ampholytes (Fig. 7) shows a large improvement over TETA ampholytes. This is also confirmed by the scan in Fig. 8. It can be seen that the ampholyte clusters, especially in the acidic region of the gel, are tighter and closer to one another, with narrower channels
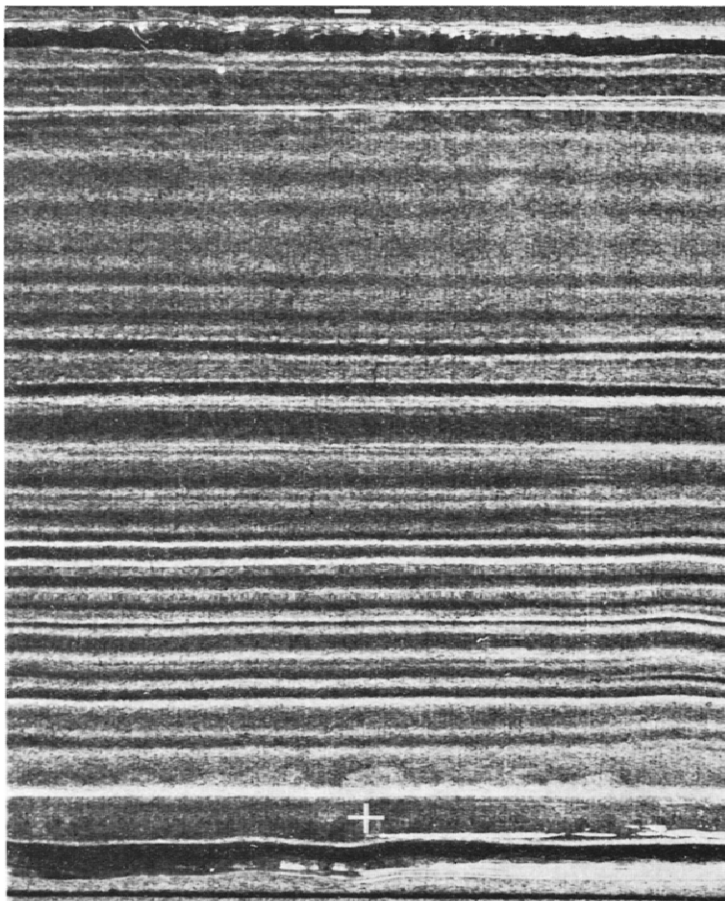
Fig. 7. TEPA ampholytes focused in a gel slab. The direction of the pH gradient is indicated by + (anode) and − (cathode). All other conditions as in Fig. 4.

in between, except for a few, wider gaps indicated by arrows in Fig. 8. The basic region of the gel, while definitely improved in comparison with TETA ampholytes, still shows the presence of less ampholyte species than the acidic region, with wider gaps and lower concentrations of individual clusters of ampholytes. This is also apparent
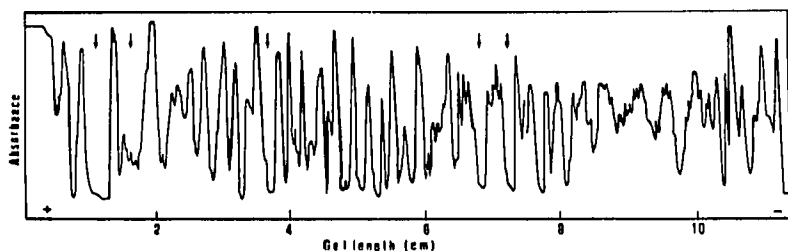


Fig. 8. Scan of focused TEPA ampholytes, from Fig. 7. The arrows indicate regions of low ampholyte concentration and the + and − signs the gel polarity. All other conditions as in Fig. 5.

in Fig. 8, which shows a progressive decrease in peak height in the region of basic ampholytes.

Fig. 9 gives the pH gradient, the conductance and the UV profiles at 280 and 360 nm obtained by focusing TEPA ampholytes in a gel slab. In comparison with Fig. 6, it can be seen that the pH gradient is smoother and that there are fewer conductivity gaps.
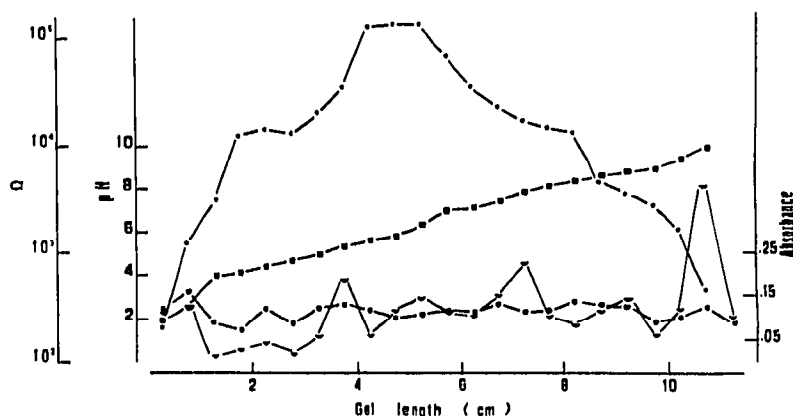


Fig. 9. Conductance (★—★) and pH (■—■) courses and UV profiles at 360 nm (▼—▼) and 280 nm (●—●) of focused TEPA ampholytes in a gel slab. All conditions as in Fig. 6.

## PEHA ampholytes

A gel slab containing 2% of PEHA ampholytes and 5% of acrylamide was polymerized and electrofocused as described above. A picture of the focused ampholytes in the gel slab is shown in Fig. 10. This represents a still greater improvement over TEPA ampholytes. The acidic regions of the two types of ampholytes appear to be rather similar, with sharp, closely spaced peaks indicating a smooth pH gradient and good conductivity in this gel region. However, the basic region of PEHA ampholytes appears to be improved in comparison with TEPA ampholytes, with high-relief, tightly packed clusters, which only towards the very basic end of the gel become progressively shallower and more spaced. There are only three major valleys of low ampholyte concentration, two towards the neutral region of the gel, and one towards the alkaline end. They are indicated by arrows in Fig. 10.

Fig. 11 gives the conductance, the pH gradient and the UV profiles at 280 and 368 nm of PEHA ampholytes focused in a gel slab. It is immediately apparent that the conductivity is much more uniform and higher than with TETA and TEPA ampholytes. There is only a small conductivity dip in the pH region 5.5. Also, the pH gradient is smoother and encompasses the pH range 3–9.5. The UV profile at 368 nm shows 6–7 peaks, representing chromophoric ampholytes whose p*I*s were given in the section *Synthesis of carrier ampholytes* and whose properties are described later.

Fig. 12 shows the separation of sickle-cell and normal haemoglobins, together with free α- and β-chains, in a polyacrylamide gel slab in the presence of 2% of PEHA ampholytes. The bands are sharply focused and their p*I*s are the same as those obtained by Perrella *et al.*[13] when using commercial Ampholine.
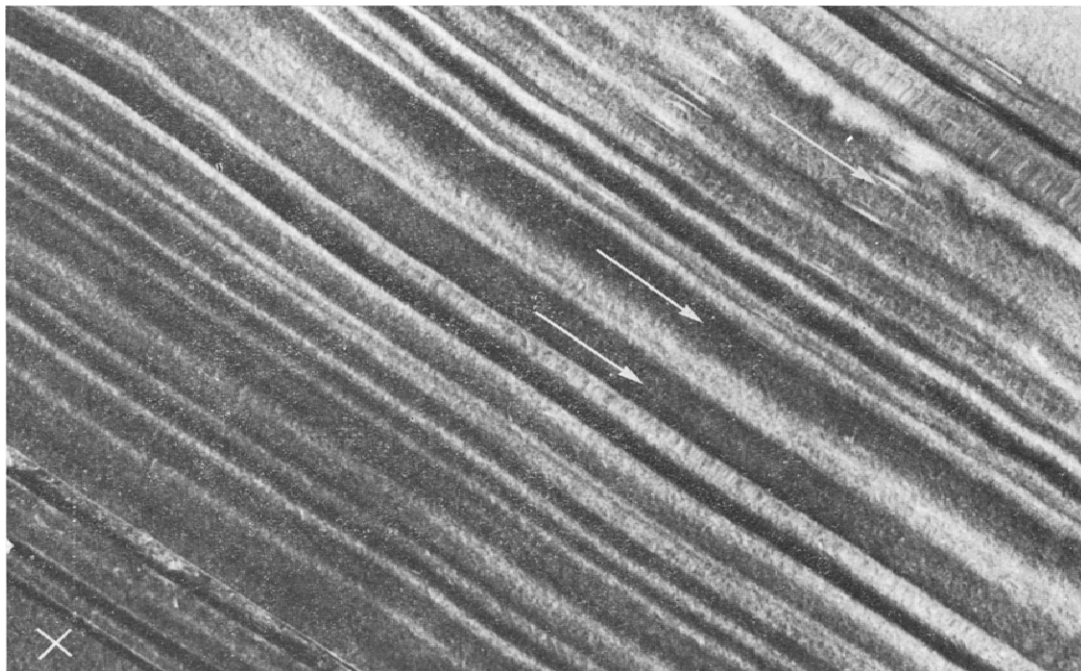
Fig. 10. Scan of focused PEHA ampholytes. The direction of the pH gradient is indicated by + (anode) and — (cathode). The arrows indicate ampholyte gaps. All other conditions as in Fig. 4.
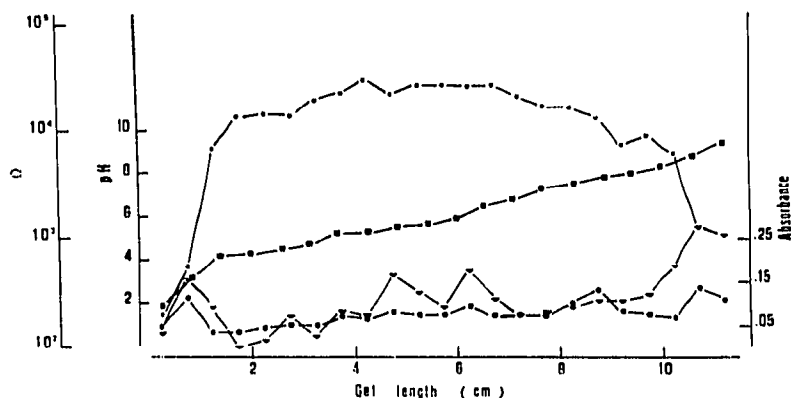


Fig. 11. Conductance (★—★) and pH (■—■) courses and UV profiles at 368 nm (▼—▼) and 280 nm (●—●) of focused PEHA ampholytes in a gel slab. All conditions as in Fig. 6.

*Some physico-chemical properties of ampholytes*

We have seen that in PEHA ampholytes, there are chromophoric structures, which, upon focusing, give 8–10 yellow bands, encompassing the pH range 3–9.5. These structures are formed during the synthesis, as PEHA and acrylic acid do not contain them (see Fig. 2). As these chromophoric compounds can sometimes represent up to 20 % of the total ampholyte mixture, and as they cannot be completely removed by charcoal treatment, we thought it worthwhile to investigate their structure.
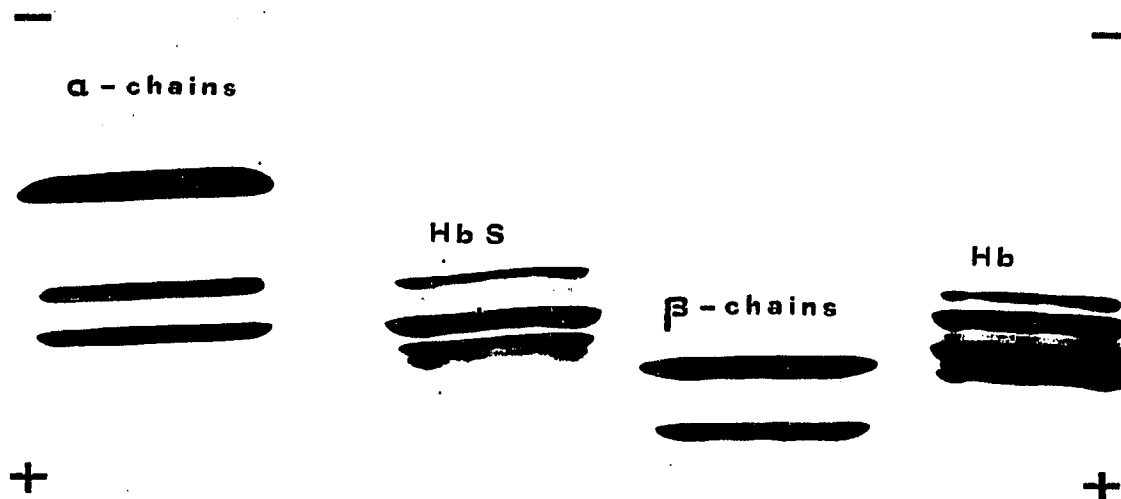
α – chains

Hb S

β – chains

Hb

Fig. 12. Isoelectric focusing of normal human adult haemoglobin (Hb), sickle-cell haemoglobin (HbS) and free α- and β-chains from haemoglobin. The run was made in a 5% acrylamide gel slab and 2% PEHA ampholytes at 2°. All the samples were equilibrated in CO. The equilibrium was obtained in 80 min with a pulsed power supply at a final voltage of 1200 V.

Fig. 13 shows the UV spectra of these chromophoric ampholytes. These spectra are identical, irrespective of whether the entire mixture or the single, isoelectric ampholytes, isolated by gel slab isoelectric focusing, are analyzed. The spectrum shows a strong peak at 368 nm, which is progressively quenched from pH 9 to pH 1. Below pH 1.8, a new peak appears at 315 nm. The two structures are in a pH-dependent equilibrium, the 315-nm peak representing the protonated and the 368-nm peak the unprotonated species. Their isosbestic point is at 335 nm. When the solution is titrated to pH 0.5 and below, allowed to remain there for a few hours and then back-titrated,
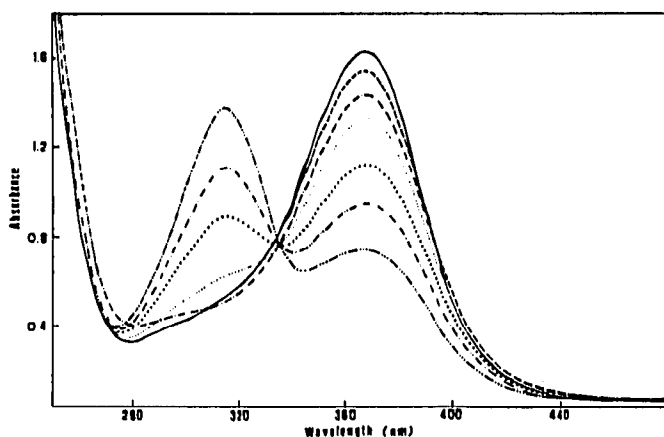
Fig. 13. UV titration spectra of "chromophoric" PEHA ampholytes. The two peaks are at 315 and 368 nm and the isosbestic point is at 335 nm. ———, pH 8.95; ---------, pH 6.8; – · – · – · –, pH 3.6; · · · · · ·, pH 1.75; ∘∘∘∘∘∘, pH 1.28; – · · – · · –, pH 1.05; – · · · – · · · –, pH 0.83.

the spectrum is completely reversible. This rules out the possibility that the 315-nm peak is an irreversible degradation product, at least during the experimental period. If we now plot the peak maxima against their respective pHs, we obtain the two "pliers"-like curves in Fig. 14, whose intersection gives the p$K$ (1.1) of the dissociating function connected with the two chromophores. This p$K$ value has not been corrected as suggested by Adrien and Serjeant[14], because even at a pH of about zero it has not been possible to extinguish the chromophore attributed to the conjugated base.
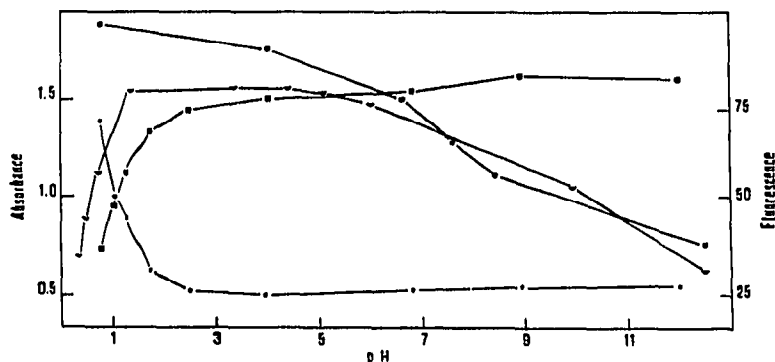


Fig. 14. pH dependence of UV and fluorescence spectra in "chromophoric" PEHA ampholytes. ★—★, UV peak at 315 nm; ■—■, UV peak at 368 nm. The intersection of these two curves gives the p$K$ (1.1) of the dissociating group linked to the two chromophores. ●—●, pH dependence of the fluorescence peak obtained by excitation at 368 nm (emission peak at 455 nm); ▼—▼, pH dependence of the fluorescence peak obtained by excitation at 315 nm (emission peak at 420 nm).

In Fig. 14 we have also plotted the fluorescence titration curves obtained by excitation at the two peak maxima. Their behaviour is in agreement with the pH dependence of the respective UV chromophores. We also measured the ORD spectra of the ampholytes, but failed to observe any rotation of polarized light in the range 500–220 nm.

We were puzzled by the very low p$K$ of the chromophore-associated function. From the known structure of ampholytes, it could be either a carboxyl group or a nitrogen function. We therefore lyophilized the chromophoric ampholytes and esterified the carboxyl group with methanol saturated with hydrogen chloride. The extent of methylation was followed with the hydroxamic acid test. However, the methylated ampholytes failed to show either a decrease of both peaks or the disappearance of one of them. We then blocked the nitrogen function with trifluoroacetic anhydride. The formation of the N-trifluoro derivative was followed by thin-layer chromatography. In this last case, both chromophores were greatly reduced, although not completely destroyed. Therefore, it appears that the two UV peaks are linked to a very weakly basic nitrogen function. Unfortunately, the p$K$ values for the amino groups in PEHA are not known. However, Vesterberg[5] reported a p$K_5$ value of 2.7 for TEPA. Judging from the progressive decrease in p$K$s in TEPA, it is reasonable to expect a p$K_6$ value in PEHA of the order of 1.1, as found in the present work. We have been able to find these chromophores even in commercial preparations of Am-

pholine from LKB (see Fig. 2 in ref. 15), which suggests a similarity between our ampholytes and the commercial product. However, during that investigation[15], we were unable to link the two chromophores and to detect the nitrogen function, because the titration studies were discontinued at pH 1.75, just when the 315-nm peak begins to appear.

The behaviour of the two UV peaks cannot be readily explained on the basis of the known structure of ampholytes, which are supposed to be polyamino polycarboxylic acids. Previously, Righetti and Drysdale[16] and Vinogradov et al.[8], on the basis of UV and fluorescence spectra and of the behaviour of ampholytes on charcoal treatment, had suggested the presence of nitrogen heterocyclic structures, formed during the synthesis. This had also been hinted at by Haglund[17] in a review paper. To test this hypothesis, we performed NMR studies on our ampholytes and on the commercial product. We chose Ampholine in the pH ranges 3–5 and 4–6 because of their strong UV spectrum and their distinct yellow colour. As shown in Fig. 15, nothing can be detected in the region of aromatic structures (6–8.6 ppm), nor is there any hint of nitrogen heterocyclic structures anywhere along the spectrum. Therefore, the structure of the two UV chromophores linked to the nitrogen function remains to be explained.
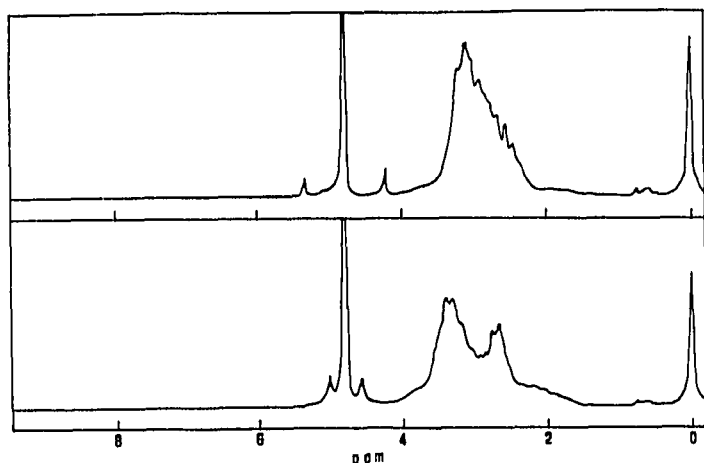


Fig. 15. NMR spectra of "chromophoric" PEHA ampholytes (above) and of LKB Ampholine, pH range 3–5 (below). The spectra were obtained with a Perkin-Elmer Model R 12 NMR spectrometer at 60 MHz in deuterium oxide.

DISCUSSION

The aim of the present investigation was to examine thoroughly the procedure for the synthesis of carrier ampholytes and to describe in more detail some of their physico-chemical properties. As already pointed out by Vesterberg[5] and Vinogradov et al.[8], it takes at least four amino groups in the polyamine to be able to synthesize ampholytes with acceptable properties. By a simple method of rendering focused ampholytes in a gel slab visible, we have been able to confirm this and to show the

actual distribution pattern of the ampholytes along the pH gradient. In going from Fig. 4 to Fig. 7 and to Fig. 10, one can see a progressive improvement in the ampholyte mixture, until in PEHA ampholytes almost all of the gaps in the gel disappear. These ampholyte patterns are well correlated with their respective conductimetric and pH profiles.

Other workers have described methods for the detection of focused ampholytes. Frater[18] reported a direct staining procedure, by precipitation in half-saturated picric acid and staining with Coomassie violet. However, by this method he was able to detect only acidic ampholytes, as the neutral and alkaline ampholytes are only poorly fixed. An interesting detection method, based on a glucose caramelization procedure on paper, was described by Felgenhauer and Pak[19]. By this technique, many more Ampholine peaks became apparent. Our detection method for ampholytes, based on differences in refractive indices, has been reported previously by Rilbe[20]. However, possibly because he used the liquid phase, the striations he was able to detect formed almost a continuous spectrum (see Fig. 3 in ref. 20). In our work, we have a pattern of sharp peaks that show up well in a densitometric scan. We realize that our method is not easily amenable to quantitation, as we can only scan a print, and therefore there is a transfer of errors from the actual gel to the negative and to the print finally used. Even so, we think that much can be inferred from a qualitative scan and that, in any event, although absolute quantitation cannot be made, the relative amounts in the various peaks can be easily appreciated. For instance, in Fig. 7 we could see a progressive flattening of the ampholyte peaks in the alkaline region. The scan in Fig. 8, in fact, demonstrates a progressive decrease in peak height, and therefore in relative amounts of basic ampholytes compared with acidic ampholytes. In any event, it is clear that what we see are not peaks of individual ampholytes, but probably, as already pointed out by Rilbe[20], clusters of carrier ampholytes. If we take that into account, and we examine the pattern complexity of Figs. 7, 8 and 10, it can be seen that Vesterberg's assumption[21] that more than 360 homologues and isomers could be generated during the synthesis, might not be too far from reality.

The reason why we investigated the properties not only of PEHA, but also of TETA and TEPA ampholytes, is that, in order to obtain a smoother pH gradient and an even conductivity, we synthesize the three types of ampholytes, and then mix them together and use this mixture as the wide ampholyte pH range, which can then be fractionated into narrow pH ranges (see the following paper). To this mixture we also add ampholytes obtained by coupling HMTA to acrylic acid. HMTA gives a very poor mixture of ampholytes, but they are clustered in the pH region 4–6, and therefore they are useful for reinforcing this pH zone.

The mixture of HMTA, TETA, TEPA and PEHA ampholytes that we have synthesized appears to be, in many respects, equivalent to the commercially available Ampholine. Perhaps a limitation is that our ampholyte mixture covers only the pH range 3–9.5, while LKB Ampholine covers the pH range 2.5–11. A great advantage, however, is that they are extremely easy to synthesize and extremely inexpensive, as the starting material is very cheap. The only difficult step in the synthetic procedure is the distillation of PEHA.

Recently, there have also been other approaches to the production of ampholytes suitable for isoelectric focusing. Thus, Pogacar and Jarecki[22] described the suitable coupling of TEPA and PEHA to either propane sulphone, vinyl sulphonate or

chloromethyl phosphonate. Their polyamino-polysulphonic ampholytes distribute into two groups, one covering the pH range 2–3.5 and the other the pH range 5.8–9.5 and there is therefore a gap in the pH region 3.5–5.8. While not useful *per se*, these "sulphonic ampholytes" could be a useful addition to the "carboxyl ampholytes" for extending their fractionation range down to pH 2.

An interesting approach has been described by Blanicky and Pihar[23], who prepared ampholytes from bactopeptone after removal of proteins by precipitation in 60% ethanol. After the removal of ethanol, a mixture of possibly several dozen different peptides remains, which appears to be rather good for isoelectric focusing. In the neutral region, the pH is improved by adding histidine. These types of ampholytes, however, are of the Rilbe type and therefore they suffer from the same inconvenience. A similar approach was used by Molnárová and Sova[25], who used casein hydrolyzates to focus different DNA species. While they were able to obtain good pH gradients down to pH 2.5, they did not present evidence to exclude binding of these peptide ampholytes to the DNA species isolated.

A completely new approach was described by Troitzki *et al.*[24]. Instead of using conventional polyamino-polycarboxylic acids or sulphonated derivatives, they used common buffers in gradients of organic solvents, such as ethanol, dioxane and glycerol, or in polyol gradients, such as mannitol, sucrose and sorbitol. Taking advantage of the $pK$ variations of these buffers in different concentrations of these solvents, they are able to generate pH gradients of approximately 1.5 pH units in different regions of the pH scale. These pH gradients are stable for up to 12 days of isoelectric focusing. They achieved separations of rabbit haemoglobin in a pH gradient of 7–8.6 obtained with borate buffer in a mixed gradient of 0–5% glycerol and 0–30% sucrose, and of human serum albumin in a pH gradient of 4.5–5.8 obtained with acetate buffer in a 0–90% glycerol gradient. It is too early to assess whether or not this system might become of general use, but it certainly deserves further investigation.

We hope that the ease with which suitable ampholytes can be synthesized and characterized in a biochemical laboratory will attract more interest to the use of isoelectric focusing on a large preparative scale, such as in continuous-flow techniques.

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